A hydrazone ligation strategy to assemble multifunctional viral nanoparticles for cell imaging and tumor targeting

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Methods

Peptide Synthesis. The peptides were synthesized manually using solid phase peptide methodology on an C-terminal amide yielding MBHA resin, using *in-situ* neutralization cycles for Boc-solid phase peptide synthesis. The arylhydrazide (4-Boc-hydrazido)terephthalic acid (Boc-HTA) was attached to the N-terminus of the peptides using a three-fold excess and TFFH (fluoro-*N,N,N',N'*-tetramethylformamidiniumhexafluorophosphate) as a coupling reagent. (5(6)-carboxyfluorescein, Sigma-Aldrich, St Louis, MO) was introduced on the side chain of lysine using DIC/HOBt (1:1) at a five-fold excess. Following chain assembly, the peptides were cleaved from the resin with HF and 10% anisole for one hr at 0 °C. The peptides were purified by HPLC. Analytical reversed-phase HPLC was performed on a Rainin HPLC system equipped with a Vydac C 18 column (10 μm, 1.0 x 15 cm, flow rate 1 mL/min). Preparative reversed-phase HPLC was performed on Waters 4000 HPLC system using Vydac C-18 columns (10 μm, 5.0 x 25 cm) and a Gilson UV detector. Linear gradients of acetonitrile in water/ 0.1% TFA were used to elute bound peptides. Peptides were characterized by electrospray ionization MS on an API-III triple quadruple mass spectrometer (Sciex, Thornhill, ON, CA). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from all of the observed protonation states of a peptide by using MacSpec software (Sciex). All observed peptide masses agreed with the calculated average masses to within 0.5 Da.

Synthesis of PEG500f. The peptide hydrazino-GK(Fluorescein)GK(PEG)-NH_2 (See Fig. S1) was synthesized using Boc-K(Fmoc)-OH. The Fmoc group was removed with 20% piperidine in DMF. Monodisperse PEG propionic acid (MW=588.7 g/mol, BioVectra) was attached using eight-fold excess and TFFH as a coupling agent.

Synthesis of VEGFR-1 antagonist peptide F56f. The original F56 peptide was modified by including two arginine residues on each end of the peptide to increase its solubility (Fig S2). A fluorescein was attached on the side-chain of an added N-terminal lysine. The peptide hydrazino-K(Fluorescein)RRGWHSDMEWWYLLGRR-NH_2 was synthesized following described procedures (Schnolzer, M., Alewood, P., Jones, A., Alewood, D. & Kent, S.B. *In situ* neutralization in Boc-

chemistry solid phase peptide synthesis.Rapid, high yield assembly of difficult sequences. *Int. J. Pept. Protein Res.***40**, 180-93 (1992).)

Derivatization of CPMV with benzaldehyde. Sulfo-succinimidyl 4-formylbenzoic acid (sulfo-SFB, Solulink, CA) in DMSO was added to CPMV (2 mg/ml in 0.1 M $NaHPO_4$, pH 7.4), 20% DMSO for 24 hr, 23°. CPMV was purified by ultracentrifugation (42,000 RPM, 3 hrs) over a 30% sucrose cushion, followed by sucrose gradient (10-40%) ultracentrifugation (28,000 RPM, 3 h) and ultrapelleting (42,000 RPM, 3 h), and resuspended in PBS. Benzaldehyde labeling was quantitated using 2-hydrazinopyridine.2 HCl (Solulink, CA) in 0.1 M acetate, pH 5, 1 h, 37° and measuring the absorbance at 350 nm ε=18,000 $M^{-1}cm^{-1}$.

Conjugation of benzaldehyde derivitized CPMV to peptides and PEG. PEG500f or F56f in 0.1 M potassium phosphate pH = 5.5, were added to benzaldehyde-modified virus (35 nM) and incubated at 37° for 4 or 12 h. For double-labeling, F56f was added first (37°, 4 h), followed by PEG500f (37°, 4 h), purified through Sephadex G-10 (Amersham), and concentrated into PBS (Ultracel PL-10, Amicon). The level of conjugation was determined by the absorbance of fluorescein at 495 nm.

Endothelial cell attachment assay. Human endothelial cell line EA.hy926 was cultured in serum-free media, PBS and purified human fibronectin (Invitrogen, CA). 15 μg diluted in PBS, was immobilized onto a 24-well plate (Nunc, NY) for 4 hr, 4°, then blocked (1% BSA, PBS) 1 hr, 37°. Cells were resuspended in medium at 10,000 cells/mL, and 0.5 mL was added to each well, incubated (3 hr, 37°, 5% CO_2) and washed (PBS, 3x). Adherent cells were fixed (3.7% formaldehyde, 15 min), stained (Hoechst33258, 30 min), gently washed (PBS, 2x) and counted using Improvision Openlab.

Fluorescence microscopy with cultured cells. Approximately 10,000 cells/well of human endothelial (EA.hy926) cells or mouse embryonal fibroblasts (MEF) cells were plated in a 12-well tissue culture

plate containing circular glass cover slips. After overnight incubation, 10 μg each of FP3, P4 or F1 was added to the media and incubated for two hrs at 37 °C, then washed with media (3x), fixed with ice-cold 4% paraformaldehyde in PBS for 10 min, washed with PBS (3x), and mounted using Prolong Gold mounting medium containing DAPI (Invitrogen). Imaging was performed using a using a 63x oil immersion lens and a Hamamatsu camera under a Zeiss Axioplan 2ieMOT microscope.

HT-29 tumor cell attachment assay and visualization. Tumors were generated in athymic nude mice (Nude-nu, Scripps Breeding Colony) using the human colon carcinoma cell line HT-29. Studies were performed in accordance with protocols approved by the Scripps Research Institutional Animal Care and Use Committee (IACUC).

Cells were mixed 1:1 with Matrigel (BD Biosciences, NJ) and injected bilaterally and subcutaneously in the flank ($1x10^6$ cells). After two weeks, animals were euthanized and tumors (~0.8 cm diameter) excised and embedded in OCT medium. Frozen sections (10 μm) were prepared on a Leica cryomicrotome. Tumor sections were fixed in 95% ethanol, 0°, 20 min. 10 μg of FP3 or P4 were added and incubated for one hr, 23°. Samples were blocked using 10% goat serum in PBS for one hr. Rabbit anti-CPMV antibody was added in 5% goat serum to PBS (45 min, 23°) and washed (PBS, 4x). Goat anti-rabbit IgGAlexaFluor 555 antibody (Invitrogen) was added in 5% goat serum to PBS (45 min, 23°, in dark). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI 1:1000 in water). Cells were washed (PBS 4x) and mounted using Vecta Shield (Vector Laboratories). Sections were imaged using a Biorad 2100 confocal microscope with a 10X or a 40X oil objective.

Interaction with tumors *in vivo*. Mice bearing HT-29 tumors (see above) were injected intravenously using 45 μg of FP3 or P4. Two hours post-injection, animals were sacrificed and tumors excised and embedded in OCT medium. Cryomicrotome sections (10 μm) were prepared and stored at -20 °C. CPMV was stained as above (using either A488 or A647 conjucated secondary antibodies. Endothelial cells were stained using a monoclonal anti-mouse CD-31 antibody (BD Biosciences) followed by a goat

anti-rat AlexaFluor647 antibody (Invitrogen). VEGFR-1 was stained using an anti-mouse VEGFR-1 antibody (R&D Systems) was followed by a goat anti-rat AlexaFluor647 antibody.

Measurement of expression of VEGFR-1 on HT-29 cells using flow cytometry. HT-29 cells were collected using Enzyme-free Hank's based Cell Dissociation Buffer (Gibco) and distributed in 96-well V-bottom plates ($1x10^6$ cells/ml). Cells were fixed with 2 % formaldehyde (PBS, 20 min 23°) and stained (monoclonal anti-mouse VEGFR-1 antibody) followed by goat anti-rat AlexaFluor647 antibody at 4° throughout. Between steps, the cells were washed consecutively with PBS, 1 mM EDTA pH 8.0; 25 mM HEPES pH 7.5; and 1 % fetal bovine serum. Cells were analyzed by FACS (Calibur, BD Biosciences, NJ). At least 10,000 events gated for single cells were collected. Triplates of each sample were measured and data were analyzed using FlowJo 8.6.3 (Tree Star, OR).

Supporting Figures

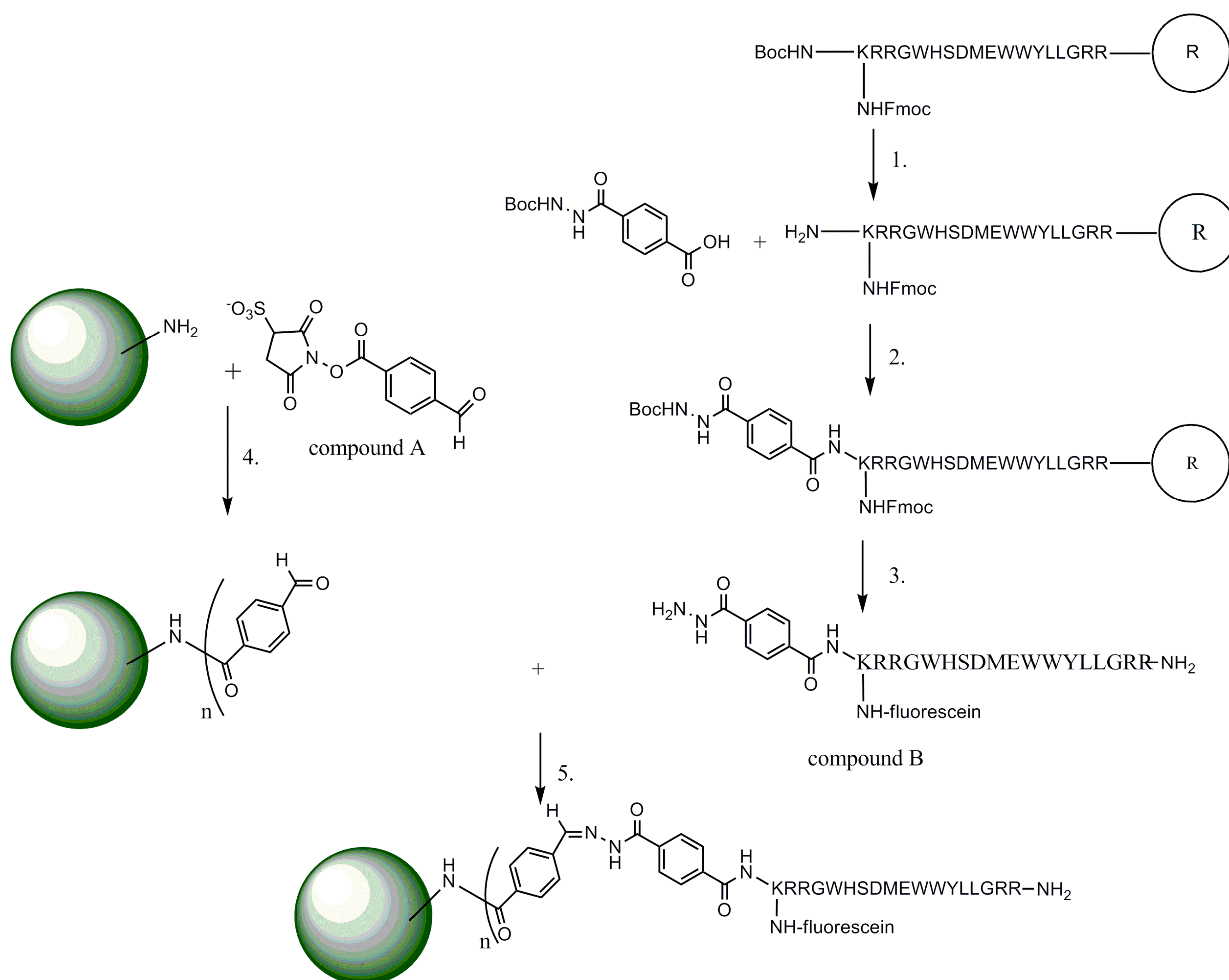

Figure S1. Synthesis of F56f and conjugation to CPMV. Right: Solid phase synthesis of F56f by Boc-SPPS. An Fmoc group on the Lys side chain enables attachment of fluorescein on the solid phase. Left: surface Lys residues are modified by compound A to introduce benzaldehyde groups onto the viral surface. Bottom: Attachement of F56f to CPMV yields a hydrazone linked product.

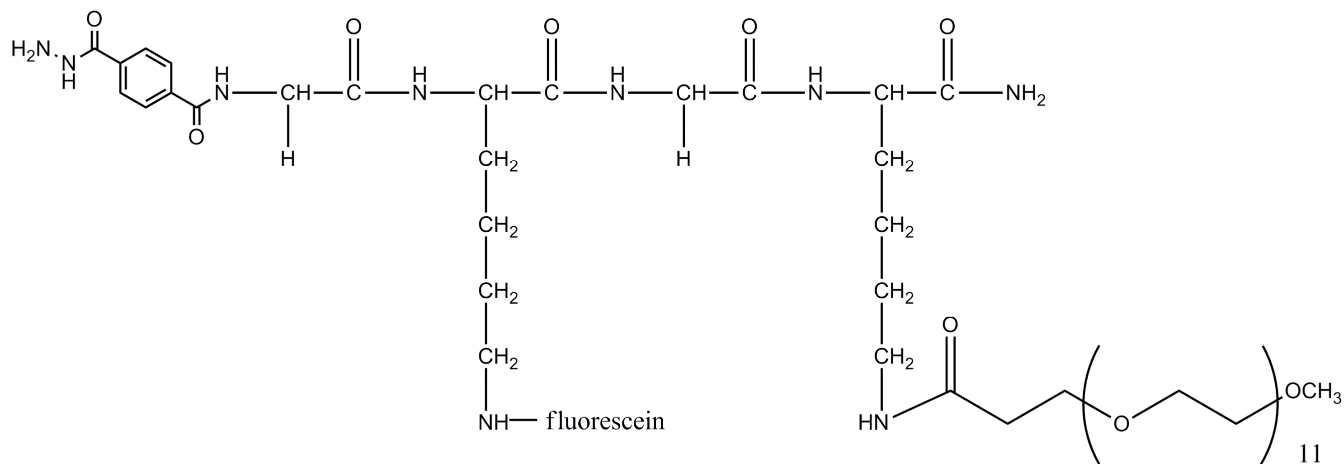

Figure S2. Structure PEG500f. The short peptide contains a hydrazide group for ligation, a mono-disperse PEG group to reduce non specific binding of the nanoparticles and fluorescein. Importantly, the fluoresceine group is placed near the conjugation site to the virus while the PEG moiety is distal to the conjugation site.